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Butanol Production From Agricultural Residues: Impact of Degradation Products on *Clostridium beijerinckii* Growth and Butanol Fermentation

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ABSTRACT: During pretreatment and hydrolysis of fiber-rich agricultural biomass, compounds such as salts, furfural, hydroxymethyl furfural (HMF), acetic, ferulic, glucuronic, ρ-coumaric acids, and phenolic compounds are produced. *Clostridium beijerinckii* BA101 can utilize the individual sugars present in lignocellulosic [e.g., corn fiber, distillers dry grain solubles (DDGS), etc] hydrolysates such as cellobiose, glucose, mannose, arabinose, and xylose. In these studies we investigated the effect of some of the lignocellulosic hydrolysate inhibitors associated with *C. beijerinckii* BA101 growth and acetone–butanol–ethanol (ABE) production. When 0.3 g/L ρ-coumaric and ferulic acids were introduced into the fermentation medium, growth and ABE production by *C. beijerinckii* BA101 decreased significantly. Furfural and HMF are not inhibitory to *C. beijerinckii* BA101; rather they have stimulatory effect on the growth of the microorganism and ABE production.

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KEYWORDS: Acetone butanol ethanol (ABE); *Clostridium beijerinckii* BA101; lignocellulosic hydrolysate; corn fiber; DDGS; fermentation inhibitors

Introduction

Acetone–butanol–ethanol (ABE) fermentation was carried out industrially throughout the United States during the first half of last century, but was discontinued in the early 1960s due to unfavorable economic conditions brought about by competition with the petrochemical industry (Ezeji et al., 2004). Fermentation substrate is an important factor influencing the cost of butanol production (Qureshi and Blaschek, 2000). Lignocellulose is the most abundant renewable resource on the planet, and has great potential as a substrate for fermentation. Hemicelluloses are the second most abundant polysaccharides in nature, and represent about 20 to 35% of lignocellulosic biomass (Koukiekolo et al., 2005). Xylan or hemicellulose may contain arabinan, galactan, glucuronic, acetic, ferulic, and ρ-coumaric acids as well as xylose. The occurrence and quantity of these compounds depend on the sources of xylan (Olsson and Hahn-Hägerdahl, 1996; Koukiekolo et al., 2005). Since substrate cost has the most influence on butanol price (Qureshi and Blaschek, 2000), we have focused our research on the use of agricultural residues for fermentative production of butanol using *Clostridium beijerinckii* BA101, a genetically modified, hyper-butanol producing strain. In particular, we are focusing on the potential use of corn stover, corn fiber, and fiber-rich distillers dried grain solubles (DDGS) as the substrates for growth and butanol production by *C. beijerinckii* BA101.

Corn fiber represents a renewable resource that is available in significant quantities from the corn dry and wet-milling industries. Approximately 4.7×10^6 dry tons of

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corn fiber is produced annually in the United States (Ebener et al. 2003). Corn fiber, depending on the source, contains about 69% fermentable sugars, of which approximately 20, 14, and 35% is in the form of starch, cellulose, and hemicellulose fractions, respectively. Typically, 2.04 kg (4.5 lb) of corn fiber is obtained from 25.40 kg (one bushel or 56 lb) of corn, which can be converted to about 1.36 kg (3.0 lb) of fermentable sugars.

Although research on genetics, fermentation, upstream processing, and downstream processing has progressed significantly, the clostridia are not able to efficiently hydrolyze fiber-rich agricultural residues. For this reason, agricultural biomass must be hydrolyzed to simple sugars using economically developed methods. Dilute sulfuric acid pretreatment can be applied to agricultural residues to bring about hydrolysis. Unfortunately, during acid hydrolysis, a complex mixture of microbial inhibitors is generated. Examples of the inhibitory compounds include furfural, hydroxymethyl furfural (HMF), and acetic, ferulic, glucuronic, ρ-coumaric acids, etc (Zaldivar et al., 1999; Varga et al., 2004).

It has been reported that the sulfate residue from sulfuric acid used for wood hydrolysis may remain at inhibitory levels in the hydrolysate (Frazer and McCaskey, 1991). As a result, the effect of sulfate on growth and ABE production by *C. beijerinckii* BA101 was also examined. Identification of butanol fermentation inhibitors and their removal or culture adaptation (to these inhibitors) may result in the successful production of butanol, a biofuel and important chemical from lignocellulosic materials such as corn fiber or DDGS. Hence, the objective of these studies was to identify butanol fermentation inhibitors present in dilute sulfuric acid lignocellulosic hydrolysates and ascertain the ability of *C. beijerinckii* BA101 to utilize mixed hexose and pentose sugars for ABE production.

Materials and Methods

Chemicals

Unless otherwise stated chemicals and laboratory media components were obtained from either Sigma chemicals (St. Louis, MO) or Fisher Scientific (Hanover Park, IL) and they were of analytical grade.

Microorganism and Culture Maintenance

Laboratory stocks of *C. beijerinckii* BA101 were routinely maintained as spore suspensions in sterile double distilled water at 4°C. *C. beijerinckii* BA101 spores (200 μl) were heat shocked for 10 min at 80°C followed by cooling in ice. The heat shocked spores were inoculated into 30 mL cooked meat medium (CMM) containing 1 g/L glucose, xylose, arabinose, mannose in the ratio of 5:4:2:1 in a 50 mL screw capped Pyrex bottle and was incubated anaerobically for 12–14 h at 35 ± 1°C. This was followed by transferring 5 mL of actively growing culture (12–14 h old) to 70 mL of tryptone–glucose–mannose–arabinose–xylose–yeast extract (TGMAXY) medium. Cells were grown anaerobically (85% N_2, 10% CO_2, 5% H_2) for 3–4 h at 35°C until the OD_{600} reached 0.8–1.0 before they were transferred into solvent production medium.

Batch Fermentation and Inhibition Studies

Pyrex screw capped bottles (175 mL) containing 120 mL medium were used throughout these studies. In all experiments the temperature was maintained at 35°C and there was no agitation or pH control. Bottles containing 55 g/L glucose, xylose, cellobiose, arabinose, mannose, galactose, or mixed sugars (glucose, xylose, arabinose, and mannose in the ratio of 5:4:2:1) and 1 g/L yeast extract were sterilized at 121°C for 15 min. For inhibition studies, sodium acetate (8.9 g/L), sodium sulfate (13.3 g/L), and various concentrations of each aldehyde or phenolic compound were introduced into the Pyrex screw capped bottles containing 55 g/L glucose and 1 g/L yeast extract. The mixture was sterilized at 121°C for 15 min. On cooling, the bottles were transferred into an anaerobic chamber (Coy, Ann Arbor, MI) at 35°C for 24 h for anaerobiosis. This was followed by the addition of filter-sterilized P2 stock solutions [(buffer: KH_2PO_4, 50 g/L; K_2HPO_4, 50 g/L; ammonium acetate, 220 g/L), (vitamin: para-amino-benzoic acid, 0.1 g/L; thiamin, 0.1 g/L; Biotin, 0.001 g/L), (mineral: $MgSO_4.7H_2O$, 20 g/L; $MnSO_4.H_2O$, 1 g/L; $FeSO_4.7H_2O$, 1 g/L; NaCl, 1 g/L)] (Qureshi and Blaschek, 1999) and inoculated with a 5% (v/v) actively growing TGY cell suspension. During the course of fermentation, 2 mL samples were collected regularly for optical density, ABE, and acid analysis.

Corn Fiber Hydrolysis and Detoxification of the Hydrolysate

Corn fiber was hydrolyzed using dilute (0.5% v/v) sulfuric acid as reported elsewhere (Ebener et al., 2003). Detoxification of the corn fiber hydrolysate (CFH) was carried out using a modified method (overliming) of Martinez et al. (2001). The pH of the hydrolysate was adjusted to 10.1 with $Ca(OH)_2$ followed by the addition of 1 g/L Na_2SO_3. The mixture was incubated in water bath for 1 h at 45°C under 100 rpm agitation. Subsequently, the precipitate (gypsum) was separated by centrifugation at 7,000*g* (4°C) for 10 min. The gypsum (41.7 ± 1.8 g/L) formed was discarded. The supernatant was neutralized to pH 6.8 with 2 M HCl and centrifuged at 4°C for 10 min at 7,000*g* to separate the precipitate. The clear supernatant was used as a carbon source to carry out the fermentation studies.

Removal of Inhibitors Using XAD-4 Resin

Inhibitors present in CFH were removed using Amberlite XAD-4 resin (Sigma Chemicals, St. Louis, MO). The resin was washed batchwise four times with deionized water to remove residual XAD-4 storage chemicals. Resin (60 g) was packed in a 1.5×51 cm^2 column (Bio-Rad, Hercules, CA). The hydrolysate was pumped into the column at a flow rate of 8 mL/min. Initial diluted solution was discarded. Approximately 400 mL CFH was treated with 60 g XAD-4 resin. By this process approximately 60–80% of furfural, HMF, and ferulic acid components were removed.

Analytical Procedures

Cell concentration was estimated by the optical density method as cell dry weight using a predetermined correlation between optical density at 540 nm wavelength and cell dry weight. ABE and acids (acetic and butyric) were measured using a 6890 Hewlett-Packard gas chromatograph (Hewlett Packard, Avondale, PA) equipped with a flame ionization detector (FID) and 1829×2 mm glass column (10% CW-20M, 0.01% H_3PO_4, support 80/100 Chromosorb WAW). Glucose concentration was determined using a hexokinase and glucose-6-phosphate dehydrogenase (Sigma chemicals, St. Louis, MO) coupled enzymatic assay as previously described (Ezeji et al., 2003). Samples were collected at various intervals for analysis of residual sugars and ABE. Concentrations of glucose, mannose, arabinose, and xylose in the glucose–mannose–arabinose–xylose mixture were determined by high performance liquid chromatography (HPLC) using an Agilent 1050 system (Palo Alto, CA). The sugars were separated on a 5 μm Supelcosil LC-NH_2, 25 cm × 4.6 mm column with 2 cm × 4.6 mm Supelcosil LC-NH_2 guard column at room temperature and detected using refractive index. A mixture of acetonitrile and deionized water (ratio 85:15) was used as the mobile phase at a flow rate of 2 mL/min. The CFH sugars were separated/analyzed by HPLC (RID-10A, Milford, MA) equipped with a refractive index detector. A HPX-87P column (Bio-Rad, Hercules, CA) was used for sugar separation performed at 80°C with Milli-Q water as the mobile phase at a flow rate of 0.5 mL/min. ABE productivity was calculated as ABE produced in g per L of broth divided by the fermentation time or the time when fermentation stopped. ABE yield was calculated as g of ABE produced per g of sugar utilized and is expressed in g/g.

Results and Discussion

Butanol Fermentation in P2 Medium Containing Individual and Mixed Sugars as Carbon Sources

Prior to carrying out CFH fermentation, investigations were made to ascertain the ability of *C. beijerinckii* BA101 to ferment representative sugars present in the lignocellulosic biomass. The sugars that were tested included glucose (control), cellobiose, mannose, arabinose, and galactose (Fig. 1). Xylose was also tested under the identical conditions. The initial sugar level was 55.0 g/L in all the experiments. In the control experiment using glucose, 17.8 g/L ABE was produced. At the end of fermentation 2.5 g/L residual acids were present in the fermentation broth. A productivity of 0.30 g/L/h was observed when using glucose as the carbon source. Fermentations with other sugars were rapid as well and they resulted in good ABE production (Fig. 1) and productivities ranging 0.23–0.32 g/L/h. The fermentations were run for 60 h at which time they all stopped producing ABE. *C. beijerinckii* BA101 produced the highest concentration of ABE (19.1 g/L) when cellobiose was used as the carbon source, while it produced the least amount of ABE when galactose (Fig. 1) was used. Dramatically elevated levels (>7 g/L) of acids were measured in batch fermentations where galactose was used as the carbon source. This observation suggests a reduced capacity for uptake and recycling of these acids by the microorganism when galactose is the carbon source. *C. beijerinckii* BA101 had a similar growth pattern in all the fermentations except in the fermentation of galactose where the microorganism experienced reduced cell growth (data not shown). Solventogenic clostridia and many anaerobes transport sugars into the cell through cell membrane using phosphoenolpyruvate-dependent phosphotransferase system (PTS) (Mitchell, 1996; Lee and Blaschek, 2001), which are involved in the transfer of a phosphate group from phosphoenolpyruvate (PEP) to the sugar. While glucose and fructose phosphorylation was supported by PEP indicating the involvement of a PTS in the uptake of these sugars, galactose appeared to be transported by a non-phosphotransferase mechanism because a significant rate of phosphorylation of this sugar was supported by ATP rather

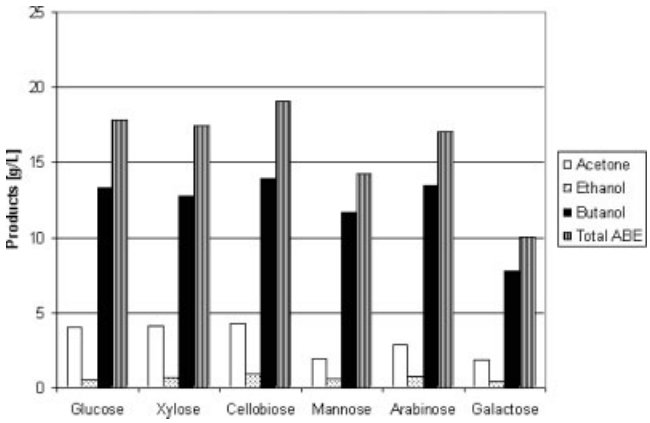

Figure 1. Production of ABE from individual sugars (55 g/L: cellobiose, glucose, galactose, mannose, arabinose, xylose) present in CFH and/or DDGS using *C. beijerinckii* BA101. Each individual sugar served as the carbon source for the P2 medium where *C. beijerinckii* BA101 was grown. Acetone butanol ethanol and total ABE concentrations were measured at the end of the fermentation.

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than PEP (Mitchell, 1996). However, *C. beijerinckii* BA101 and *C. beijerinckii* 8052 have been shown to involve both transport systems simultaneously in the uptake of glucose during fermentation with the non-PTS system (ATP-dependent glucokinase) predominant during the solventogenic phase when the PTS is repressed (Lee and Blaschek, 2001; Lee et al., 2005). Therefore, it is likely that galactose uptake must occur by a non-PTS mechanism and its phosphorylation catalyzed by galactokinase. Efficient galactose uptake may depend on the expression of galactokinase genes and the activity of galactokinase enzymes (ATP-α-D-galactose-1-phosphate transferase (galactokinase), uridine diphosphoglucose-D-galactose-l-phosphate uridylyl transferase, and uridine diphosphogalactose-4-epimerase) during the solventogenic growth phase when PTS is repressed. Similarly, galactose uptake in *C. pasteurianum* has been shown to occur by a non-PTS mechanism where the phosphorylation of galactose is catalyzed by galactokinase (Booth and Morris, 1975; Daldal and Applebaum, 1985).

The results obtained from fermentation of different sugars showed that *C. beijerinckii* BA101 is capable of utilizing products of lignocellulosic biomass hydrolysates efficiently for the production of ABE. This characteristic applies to *C. beijerinckii* 8052, although *C. beijerinckii* BA101 has higher sugar utilization and solvent formation capacity than the parent strain. It should be noted that the glucokinase activity associated with *C. beijerinckii* BA101 was greater than for *C. beijerinckii* 8052 and this characteristic may explain why *C. beijerinckii* BA101 has better glucose utilization and greater solvent formation ability than *C. beijerinckii* 8052 (Lee et al., 2005). However, fermentation results of different sugars did not indicate that *C. beijerinckii* BA101 is capable of utilizing mixed sugars for ABE production. In order to examine the ability of *C. beijerinckii* BA101 to utilize mixed sugars (hexoses and pentoses) for ABE production, an experiment was carried out where sugars such as glucose, mannose, arabinose, and xylose (GMAX) were added in the ratio of 5:1:2:4 in the medium prior to being fermented using *C. beijerinckii* BA101. After 84 h of fermentation (when fermentation stopped), 17.9 g/L ABE was produced, with a maximum of 13.9 g/L butanol (Fig. 2A). Although butanol and total ABE produced in this fermentation are similar to the glucose control (Fig. 1), the fermentation time was longer (84 h; than the control, 60 h) resulting in a lower productivity (0.21 g/L/h).

Throughout the fermentation, low levels of acids were present in the fermentation medium (Fig. 2A), which indicates an efficient fermentation. All the sugars (glucose, mannose, arabinose, and xylose) were concurrently utilized during the fermentation, although the rate of sugar utilization was sugar specific (Fig. 2B). The simultaneous uptake and metabolism of these sugars for ABE production is a very desirable feature since the sugar composition of lignocellulosic biomass hydrolysates such as corn fiber and DDGS is diverse. The rapid glucose utilization is not surprising since glucose represents the preferred substrate in

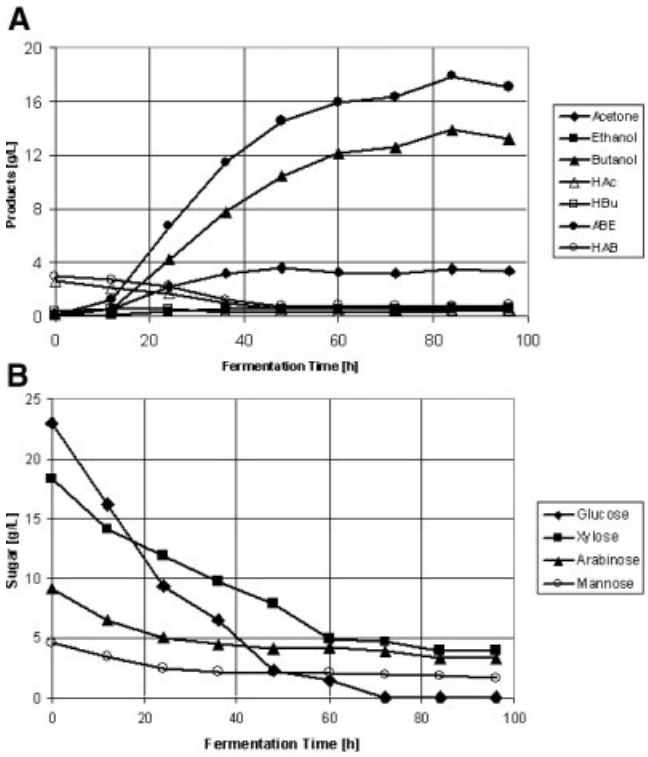

Figure 2. Production of ABE from mixed sugars [glucose, mannose, arabinose, xylose (GMAX) in the ratio of 5:1:2:4] present in CFH and/or DDGS by *C. beijerinckii* BA101. The mixed sugar was the carbon source for the P2 medium. **A**: Fermentation time versus products; **B**: Fermentation time versus sugars. HAc-acetic acid, HBu-butyric acid; HAB-acetic acid plus butyric acid.

the metabolism of many microorganisms. After glucose exhaustion (Fig. 2B), the next sugar of choice for *C. beijerinckii* BA101 was not apparent, but on a percentage basis, xylose was the next sugar utilized in high amounts after glucose. The order of sugar preference by *C. beijerinckii* BA101 can be summarized as glucose > xylose > arabinose > mannose. At the end of fermentation, 0 g/L glucose, 4.0 g/L xylose, 3.3 g/L arabinose, and 1.7 g/L mannose remained unutilized due to ABE toxicity to the culture. In a closely related study, batch fermentation of the glucose/xylose mixture by *C. acetobutylicum* 824 on a complex medium showed that this bacterium metabolizes glucose first and rapidly before utilizing xylose for ABE production (El Kanouni et al., 1998). Results presented in Figures 1 and 2 suggested that *C. beijerinckii* BA101 can utilize lignocellulosic sugars simultaneously.

Effect of Detoxification on the Fermentability of CFH

In our previous reports on the production of butanol from CFH, we demonstrated that dilute sulfuric acid CFH hydrolysate was toxic to *C. beijerinckii* BA101 (Ebener et al., 2003). As a result of toxicity, poor cell growth and reduced fermentation occurred. XAD-4 has been demonstrated to be

an effective polymeric adsorbent to remove microbial inhibitory aldehydes such as furfural from lignocellulosic hydrolysates (Weil et al., 2002), but the efficacy of this column with other inhibitory compounds such as ferulic, glucuronic, and ρ-coumaric acids, etc. has not been ascertained. The XAD-4-treated CFH resulted in the production of 8.8 g/L ABE as compared to 17.8 g/L in the control experiment when using glucose as the carbon source (Qureshi et al., unpublished). In addition, CFH was detoxified using overliming procedure, partly due to economic reasons and partly to investigate the effect of this treatment on cell growth and fermentation. The *C. beijerinckii* BA101 culture was grown in glucose-based P2 medium (control) and CFH treated with $Ca(OH)_2$ and XAD-4-based P2 medium (Fig. 3A). In the control experiment a cell concentration of 2.34 g/L was obtained after 48 h of fermentation. A cell concentration of 1.67 g/L was obtained in CFH fermentation treated with $Ca(OH)_2$. Cell growth in XAD-4-treated CFH was poor. The concentrations of individual sugars that were present in CFH (before and after treatments and after fermentation) are shown in Table I. It should be noted that fermentable sugars have been reported to be unaffected or stable during overliming treatment procedure (pH 10, temperature below 45°C, and time 90 min; Purwadi et al. 2004).

The ABE production profiles by *C. beijerinckii* BA101 using $Ca(OH)_2$-treated CFH medium over the course of 88 h showed that the culture produced 7.4 g/L acetone, 7.2 g/L butanol, and 1.0 g/L ethanol resulting in a total ABE concentration of 15.6 g/L (Fig. 3B). The culture performed better in $Ca(OH)_2$-treated CFH than it did with XAD-4-treated CFH. The total acids present at the beginning of the fermentation of $Ca(OH)_2$-treated CFH was 7.0 g/L which decreased to 4.2 g/L during the course of fermentation. This suggests that there was obvious reassimilation of acids (acetic and butyric) by *C. beijerinckii* BA101. In contrast, there was more residual acids (above 8 g/L) present in XAD-4-treated CFH fermentation broth (after the fermentation was over) than in the $Ca(OH)_2$-treated CFH medium. The ABE results obtained in this fermentation medium [CFH treated with $Ca(OH)_2$] are encouraging but the acetone–butanol ratio was increased from the normal 1:2.5 to 1:1, which is uncharacteristic of this culture. Although different substrates have been reported to influence the acetone–butanol ratio (Maddox, 1980), it is not clear at this point whether residual inhibitory compounds and the initial high concentration of acetic acid in the overlimed CFH hydrolysate played a role in the redirection of carbon toward acetone production by *C. beijerinckii* BA101. At the end of the fermentation, the residual sugar concentration was greater in the XAD-4-treated CFH fermentation broth than in the $Ca(OH)_2$-treated CFH fermentation broths (Table I) although the initial total sugar concentration in the later was higher (Table I). This shows that the sugar utilization was significantly greater in the $Ca(OH)_2$-treated CFH fermentation than in the XAD-4-treated CFH fermentation.

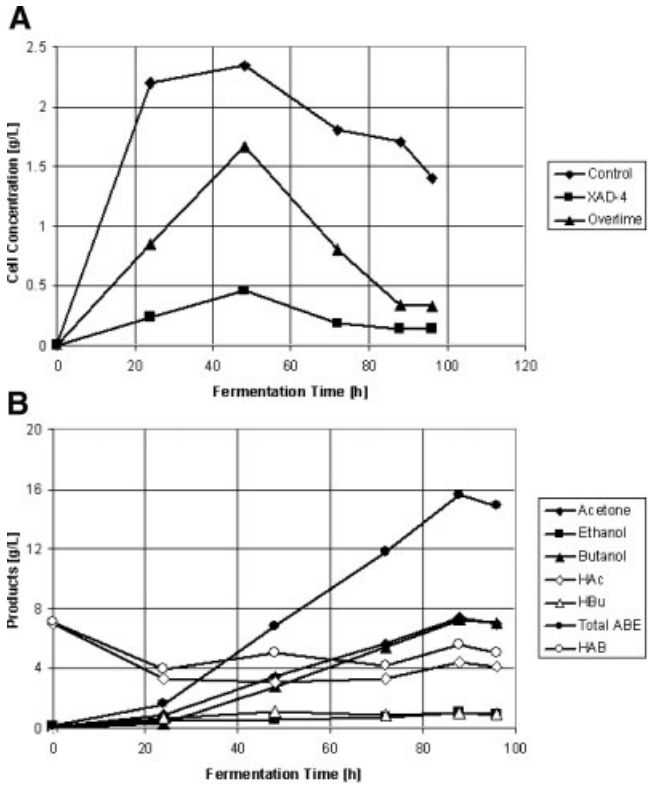

Figure 3. Effect of overliming and XAD-4 treatment of CFH on cell growth and ABE production by *C. beijerinckii* BA101. XAD-4 and overlimed-treated CFH were used as sole carbon source for P2 medium. **A**: Fermentation time versus cell concentration; (**B**) fermentation time versus products. For HAc, HBu, and HAB see Figure 2 legends.

Table I. Effect of detoxification procedures on the sugar concentration present in CFH before and after fermentation.

	CFH composition before fermentation (g/L)			Residual sugars after 96 h of fermentation (g/L)	
Sugars	Untreated CFH	$Ca(OH)_2$-treated CFH	XAD-4-treated CFH	$Ca(OH)_2$ treated	XAD-4 treated
Glucose	20.9	20.1	19.0	0.0	0.7
Xylose	18.8	18.3	17.8	7.2	15.3
Arabinose	12.2	12.0	10.5	6.5	8.5
Galactose	3.2	3.2	2.2	2.0	1.9
Mannose	1.8	1.6	1.3	1.3	1.2
Total	56.9	55.2	50.8	17.0	27.6

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Butanol Fermentation in Medium with Added Sodium Acetate and Sodium Sulfate

In order to determine the toxicity of acetate and sulfate on *C. beijerinckii* BA101 during butanol fermentation, the effect of these compounds was investigated. Acetate is produced when the hemicellulose part of the lignocellulosic biomass is hydrolyzed, while sulfate is a result of sulfuric acid used for the treatment of lignocellulosic biomass. Based on a material balance it was calculated that the sulfuric acid hydrolyzed CFH contained 8.9 g/L sodium acetate and 13.3 g/L sodium sulfate equivalent. It has been demonstrated that the presence of salts in the fermentation medium inhibits cell growth and fermentation by *C. acetobutylicum* (Maddox et al., 1995).

Cell growth of *C. beijerinckii* BA101 in the presence of 8.9 g/L sodium acetate (CH_3COONa) and 13.3 g/L sodium sulfate (Na_2SO_4) is shown in Fig. 4A. When compared to the control (glucose, Fig. 3A), cell growth in the presence of sodium acetate was slightly improved, while in the presence of sodium sulfate it was poor (Fig. 4A). In the control experiment a cell concentration of 2.34 g/L was achieved in 48 h while in the presence of sodium acetate it was 2.45 g/L. In the presence of sodium sulfate a maximum cell concentration of 1.09 g/L was obtained after a period of 48 h. Acetate inhibited the growth of ethanologenic *Escherichia coli* LY01 by about 35% when the fermentation medium contained 8.0 g/L acetate (Zaldivar and Ingram, 1999). In our studies, the maximum cell concentration achieved by *C. beijerinckii* BA101 was 53% lower in the presence of 13.3 g/L sulfate and 14% higher in the presence of sodium acetate than the maximum achieved in the control. The maximum ABE produced in the presence of 8.9 g/L acetate was 20.3 g/L (Fig. 4B), which is 14% higher than the maximum achieved in the control (Fig. 1). The individual levels of acetone, butanol, ethanol, acetic acid, and butyric acid after 72 h were 4.1, 0.5, 15.7, 2.2, and 1.2 g/L, respectively.

The fermentation carried out in the presence of 13.3 g/L sodium sulfate was allowed to run for 84 h (Fig. 4C). Production of ABE stopped after 72 h, at which time 13.3 g/L ABE (acetone 3.2, ethanol 0.4, and butanol 9.7 g/L) were present in the broth. The culture produced 4.3 g/L acetic acid and 1.9 g/L butyric acid thus totaling 6.2 g/L acids. The total ABE concentration that was achieved in this system was much less than that achieved in the control experiment (without sodium sulfate −17.8 g/L). This suggested that the presence of sodium sulfate in the CFH medium was one of the factors that contributed to the inhibition of cell growth and ABE production by *C. beijerinckii* BA101 when sulfuric

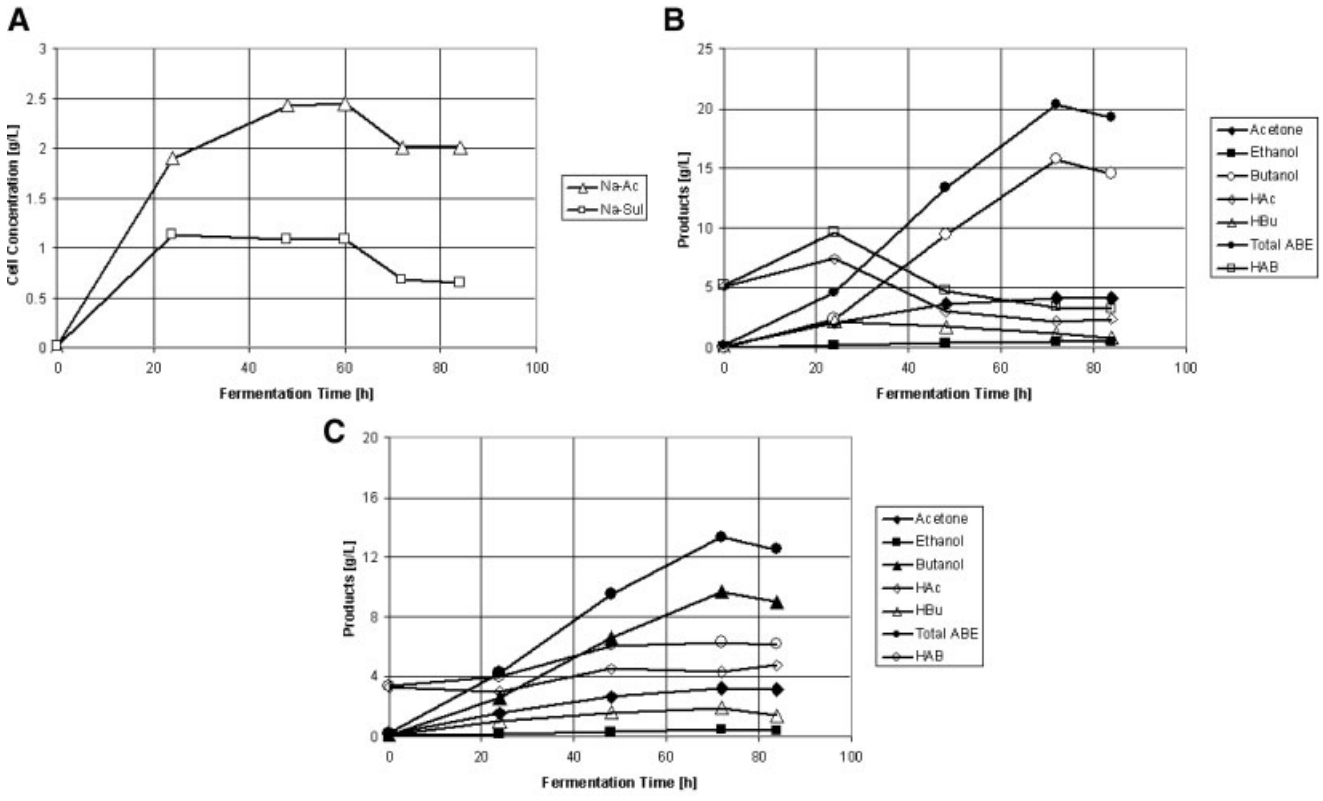

Figure 4. Effect of 8.9 g/L sodium acetate and 13.3 g/L sodium sulfate on cell growth of *C. beijerinckii* BA101 and ABE production. **A**: Fermentation time versus cell concentration when *C. beijerinckii* BA101 was cultivated in P2 medium containing 8.9 g/L Na–Ac and 13.3 g/L Na–Sul. **B**: Fermentation time versus products for sodium–acetate (Na–Ac) treatment. **C**: Fermentation time versus products for sodium-sulfate (Na-Sul) treatment. For HAc, HBu, and HAB see Figure 2 legends.

acid CFH was used as a substrate. When *C. beijerinckii* BA101 was cultivated in P2 medium containing 8.9 g/L sodium acetate plus 13.3 g/L sodium sulfate, the culture produced a total of 11.3 g/L ABE (even lower concentration), suggesting that the combination of sodium acetate and sodium sulfate was more toxic to *C. beijerinckii* BA101 than the effect of sodium sulfate alone.

Butanol Fermentation in Medium with Added Representative Inhibitory/Degradation Compounds

During sulfuric acid hydrolysis of lignocellulosic biomass such as corn fiber and DDGS, products such as furfural, HMF, syringaldehyde, glucuronic acid, ρ-coumaric acid, ferulic acid, and some other phenolic compounds are generated. During fermentation, these chemicals may affect the culture negatively. In order to investigate the impact of these products on growth of *C. beijerinckii* BA101 and fermentation, these chemicals were added to the P2 medium and cell growth and fermentation were monitored. In order to determine the compounds responsible for inhibition (other than salts that have been discussed above) of *C. beijerinckii* BA101 in CFH fermentation and why the culture did not produce as much ABE from XAD-4- and $Ca(OH)_2$-treated CFH as it did in the glucose control, different concentrations (0.3–3.0 g/L) of the above-mentioned inhibitory compounds were incorporated into the medium, and their effects on growth, sugar utilization, and ABE production were investigated.

Growth of *C. beijerinckii* BA101 was stimulated by furfural and HMF (Fig. 5A). Interestingly, the maximum cell concentration recorded was 2.66 g/L when *C. beijerinckii* BA101 was grown in medium containing 2.0 g/L HMF, which was 14% higher than the maximum achieved in the control. At 2.0 g/L furfural, the maximum cell concentration of *C. beijerinckii* BA101 in the fermentation broth was 2.50 g/L, which is approximately 7% higher than the maximum achieved in the control. *C. beijerinckii* BA101 grew well in the presence of syringaldehyde at levels as high as 1.0 g/L (Fig. 5A). However, syringaldehyde has been shown to reduce the growth of *E. coli* KO11 and *E. coli* LY01 by more than 75% during the first 24 h post-inoculation and by 40% after 48 h of growth (Zaldivar et al., 1999). In our studies, ferulic acid was the most toxic compound tested, followed by ρ-coumaric acid and both acids completely inhibited *C. beijerinckii* BA101 when their concentrations exceeded 1.0 g/L (Fig. 5A). We observed that the effect of these acids on *C. beijerinckii* BA101 cell growth seemed to increase with fermentation time and there was sudden termination of cell growth when the fermentation reached the solventogenic phase. It is important to note that during medium preparation, ferulic acid was observed to be insoluble in water while ρ-coumaric acid was sparingly soluble but both (at the concentrations tested) became soluble after sterilization of the media in the autoclave.

The toxicity of the organic acids present in lignocellulosic biomass hydrolysates has been correlated with their degree

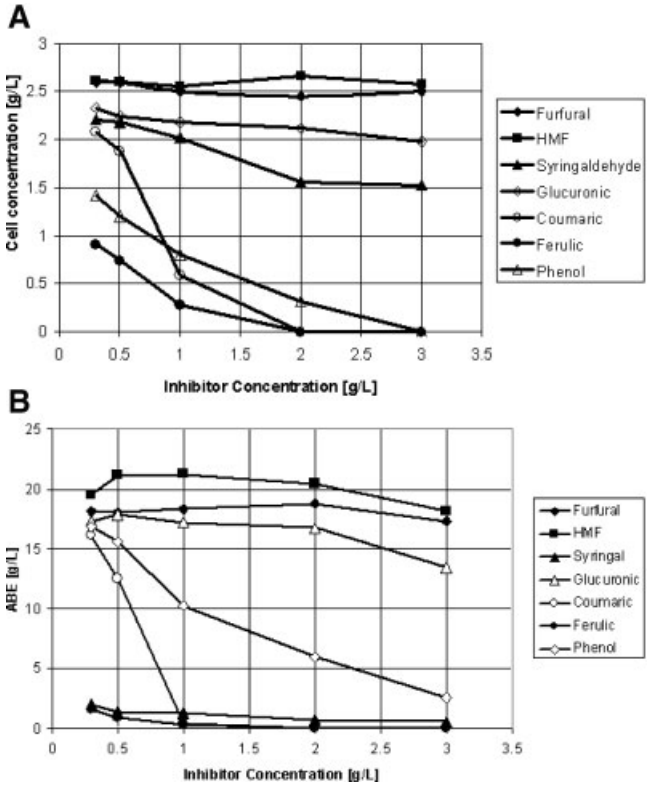

Figure 5. Effect of representative inhibitors generated during dilute sulfuric acid hydrolysis of corn fiber and/or DDGS on cell growth of *C. beijerinckii* BA101 and ABE fermentation. **A**: Cell concentration; **B**: ABE concentrations.

of hydrophobicity suggesting that toxicity involves a hydrophobic target including but not limited to the cell membrane (Zaldivar and Ingram, 1999; Zaldivar et al., 1999). Little or no ABE was produced by *C. beijerinckii* BA101 during batch fermentation in the presence of 0.3 g/L ferulic acid (Fig. 5B). ρ-Coumaric acid (0.5 g/L) inhibited ABE production by *C. beijerinckii* BA101 by 30% (Fig. 5B). Ferulic and coumaric acids are phenolic acids and phenolic compounds have long been known to be toxic to bacterial cells. It is likely that lethal concentrations of ferulic and ρ-coumaric acids may inhibit *C. beijerinckii* BA101 by damaging the hydrophobic sites of the bacterial cells. This is because phenolic compounds cause increase in membrane fluidity, a property known to affect membrane permeability (Heipieper et al., 1994) causing leakage of cellular contents. At a sublethal phenol concentrations, bacterial cells have been shown to respond by modifying the fatty acid (saturation) composition of their lipids and this increase in the degree of saturation of lipids probably compensated for the increase of fluidity of the membrane induced by the phenols (Keweloh et al., 1991).

On the other hand, ABE production by *C. beijerinckii* BA101 was stimulated by the presence of furfural and HMF (Fig. 5B). When 0.5–1.0 g/L HMF was added to the fermentation medium, maximum ABE concentration was

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21.2 g/L, which is approximately 19% higher than the maximum achieved in the control (Control Fig. 1). At 2.0 g/L furfural and HMF, *C. beijerinckii* BA101 produced 18.8 g/L and 20.4 g/L ABE, respectively, which are approximately 6 and 15% higher than the maximum achieved in the control experiment. Enteric bacteria and yeast have been shown to metabolize furfural (Boopathy et al., 1993) and HMF (Wang et al., 1994). It is not clear why *C. beijerinckii* BA101 experienced elevated growth and ABE production (Fig. 5A and B) in the presence of furfural and HMF, but having the capacity to metabolize furfural and HMF is likely. Furthermore, at a concentration of 2.0 g/L glucuronic acid, maximum ABE concentration achieved in the fermentation broth was approximately 6% less than the control. It should be noted that the syringaldehyde (1.0 g/L) which had minimal effect on *C. beijerinckii* BA101 growth especially before the solventogenic growth phase was a potent inhibitor for ABE production even at concentrations as low as 0.3 g/L (Fig. 5B). The mode of syringaldehyde selective inhibition on ABE production (as opposed to cell growth) by *C. beijerinckii* BA101 is not known at this time. It is possible that syringaldehyde interferes with the reactions involved in the glycolytic pathway and alcohol dehydrogenase enzymes secretion, which are responsible for the production of a variety of products including ABE. Preliminary investigation in our laboratory showed that there was a twofold decrease in the expression of NADH-dependent butanol dehydrogenase BDHII and a 7–11-fold increase in the stage V sporulation genes in *C. beijerinckii* during fermentation in the presence of syringaldehyde (data not shown).

In the presence of furfural (0.3–3.0 g/L) in the medium, the residual glucose level ranged from 9.3 to 10.1 g/L, while in the presence of HMF it was 4.2–8.6 g/L. It is obvious that *C. beijerinckii* BA101 utilized the glucose for cell growth and ABE production. In the presence of syringaldehyde (0.3–3.0 g/L) the residual sugar level ranged from 35.3 to 45.3 g/L, respectively. From the cell growth and ABE production results it is clear that most of the sugar was used for cell growth and cell maintenance as little or no ABE was produced during the fermentation. Ferulic acid was as toxic to ABE production as syringaldehyde and little or no ABE was produced. In the fermentation where inhibition due to ferulic acid was studied within the same concentration range (0.3–3.0 g/L), the level of residual glucose was 49.7–55.8 g/L. In the present studies, data on cell growth, ABE production, and the amount of residual glucose demonstrated that some of these chemicals were toxic to cell growth, while others were toxic to both growth and ABE production. ABE yields obtained in the presence of different inhibitors are shown in Table II.

It was subsequently investigated if adding more than one inhibitor at a time to the fermentation medium affected cell growth, ABE production, or ABE productivity. In run I (Table III), 1 g/L of each of furfural and HMF were added to the medium, while in run II (Table III) the furfural and HMF concentration was increased to 1.5 g/L each. In run I a maximum cell concentration of 2.2 g/L was achieved after 48 h of growth, while in run II it was 2.3 g/L (Table III). In control fermentation (no inhibitors added) a cell concentration of 2.34 g/L was recorded (Fig. 3A). In the two fermentations (run I and run II), cell concentrations were reduced by 7 and 5%, respectively. In the next run (III), 0.67 g/L each of furfural, HMF, and glucuronic acid were added while in run IV their concentrations were increased to 1.0 g/L each (Table III). In these runs (III and IV) maximum cell concentrations of 2.1 and 2.0 g/L were achieved, respectively. These values were reduced by 10 and 13%, respectively. A comparison of cell concentrations in the four runs with control (Fig. 3A) and experiments where inhibitors were tested individually suggests that the combinations of inhibitors were toxic to the culture. The lowest cell concentration was obtained in run IV where the three inhibitors were mixed at 1.0 g/L each.

Table II. ABE yields obtained when *C. beijerinckii* BA101 was cultivated in P2 medium containing following inhibitors.

	Inhibitor concentration and ABE yield		
Compound	Inhibitor conc.(g/L)	Cell concentration (g/L)[a]	ABE yield (g/g)
Control	0.0	2.25	0.39
Furfural	2.0	2.45	0.40
HMF	1.0	2.55	0.43
Glucuronic acid	0.5	2.24	0.41
ρ-Coumaric acid	0.3	2.08	0.38
Syringaldehyde	0.3	2.21	0.10
Phenol	0.3	2.2	0.42
Ferulic acid	0.3	0.91	ND*

*ND, not determined due to little or no ABE production.
[a]Maximum cell concentration attained at the indicated inhibitor concentration.

Table III. Synergistic effect of mixture of inhibitors on cell growth of *C. beijerinckii* BA101 and ABE production using glucose-based P2 medium.

Parameters	Run I	Run II	Run III	Run IV
Acetone (g/L)	3.7	4.2	3.7	3.8
Butanol (g/L)	13.7	13.8	13.1	13.9
Ethanol (g/L)	0.5	0.5	0.5	0.6
ABE (g/L)	17.9	18.5	17.3	18.3
Total acids (g/L)[a]	1.6	1.4	1.5	1.1
Initial glucose (g/L)	55.1	55.8	54.8	55.9
Residual glucose (g/L)	9.3	9.1	9.8	9.9
Cell concentration (g/L)[b]	2.2	2.3	2.1	2.0
ABE yield (g/g)	0.39	0.40	0.39	0.40
Productivity (g/L.h)	0.25	0.26	0.21	0.22
Fermentation time (h)	72	72	84	84

[a]Total acids measured at the fermentation time.
[b]Maximum cell concentration reached.
For control run: See Fig. 1A, initial glucose 55.6 g/L, yield 0.39 g/g, maximum cell concentration 2.34 g/L, fermentation time 60 h. Control medium did not contain inhibitor.
Run I: Furfural 1.0 g/L + HMF 1.0 g/L.
Run II: Furfural 1.5 g/L + HMF 1.5 g/L.
Run III: Furfural 0.67 g/L + HMF 0.67 g/L + glucuronic acid 0.67 g/L.
Run IV: Furfural 1.0 g/L + HMF 1.0 g/L + glucuronic acid 1.0 g/L.

In run I (Table III), 17.9 g/L ABE was produced which is comparable to the control experiment (17.8 g/L). In this run (I), 1.6 g/L total acids were produced. In the run II where furfural and HMF were added at a concentration of 1.5 g/L each, 18.5 g/L ABE and 1.4 g/L acids were produced. Run III had 0.67 g/L each of furfural, HMF, and glucuronic acid and it produced 17.3 g/L ABE and 1.7 g/L acids. The individual levels of ABE concentrations, initial glucose concentrations, fermentation times, and ABE yields obtained in the four runs are presented in Table III. Run IV resulted in the production of 18.2 g/L ABE and 1.2 g/L total acids. It should be noted that cell concentrations in the four runs were lower than the control and the amounts of ABE produced were within ±3–4% of the control. However, ABE concentration was lower than the 21.2 g/L achieved when 1 g/L HMF alone was added to the culture (Fig. 5B), suggesting that the stimulatory effect of HMF disappeared when a combination of inhibitors was used. The amounts of residual acids in the four runs were low ranging from 1.2 to 1.7 g/L (Table III). ABE productivity in Run I was 0.25 g/L/h, which is lower than that achieved in the control experiment (0.30 g/L/h). The productivities obtained in run II to IV were 0.26, 0.21, and 0.22 g/L/h, respectively (Table III). A comparison with the control (without inhibitor) suggests that cell growth and productivities by *C. beijerinckii* BA101 were negatively affected due to the combined effect of the inhibitory compounds. As a result of the combined effects of the inhibitory components, no strong signs of stimulatory action due to furfural or HMF were observed. This observation is different from results obtained with *E. coli* fermentations carried out in the presence of these compounds, which showed that combinations of furfural with other aldehydes were more than additive in toxicity for *E. coli* growth (Zaldivar et al., 1999).

Conclusions

Among the different sugars tested for ABE production by *C. beijerinckii* BA101, glucose is the preferred substrate although this culture utilizes xylose, arabinose, mannose, and galactose. During the fermentation of mixed sugar (GMAX), all the sugars were utilized concurrently throughout the fermentation, although the rate of sugar utilization was sugar specific. The concurrent uptake and metabolism of these sugars is a desirable feature since the long-term objective of this work is to use *C. beijerinckii* BA101 to ferment corn fiber and DDGS hydrolysates. These hydrolysates contain glucose, xylose, arabinose, galactose, and mannose. Furfural and HMF (3g/L) are not inhibitory to *C. beijerinckii* BA101, rather they are stimulatory and the mixture of the two affects the culture negatively. *C. beijerinckii* BA101 is not inhibited by acetates; instead higher solvents are produced by the culture under high concentrations of acetate in the medium. Alternatively, syringaldehyde, ferulic, and ρ-coumaric acids were potent inhibitors of ABE production by *C. beijerinckii* BA101. Because of the presence of these inhibitors in CFH, fermentation of untreated CFH is not possible. In addition, we have demonstrated that salts generated during dilute sulfuric acid hydrolysis are toxic to *C. beijerinckii* BA101.

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